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p300 and p300/cAMP-responsive Element-binding Protein Associated Factor Interact with Human T-cell Lymphotropic Virus Type-1 Tax in a Multi-histone Acetyltransferase/Activator-Enhancer Complex*

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Robert Harrod‡, Yu-Liang Kuo‡, Yong Tang‡, Yao Yao‡, Alex Vassilev§, Yoshihiro Nakatani§, and Chou-Zen Giam‡¶

From the ‡Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814 and the §Laboratory of Molecular Growth Regulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892-2753

The human T-cell lymphotropic virus, type (HTLV)-1 trans-activator, Tax, coordinates with cAMP-responsive element-binding protein (CREB) and the transcriptional co-activators p300/CBP on three 21-base pair repeat elements in the proviral long terminal repeat (LTR) to promote viral mRNA transcription. Recruitment of p300/CBP to the activator-enhancer complex, however, is insufficient to support Tax-dependent LTR trans-activation. Here, we report that the p300/CBP-associated factor (P/CAF) is a critical and integral component of the functional HTLV-1 activator-enhancer complex. The HTLV-1 Tax protein directly binds P/CAF *in vitro* and co-immunoprecipitates with this co-activator *in vivo*. The Tax mutants (K88A and V89A) defective for p300/CBP-binding and LTR trans-activation, retained their abilities to interact with P/CAF. The M47 mutant (L319R, L320S) protein, which has previously been shown to interact with p300/CBP, by contrast, failed to form complexes with P/CAF and is impaired in LTR trans-activation. Furthermore, LTR trans-activation by Tax is competitively inhibited by the adenoviral E1A 12S gene product, which displaces P/CAF from p300/CBP and inhibits the histone acetyltransferase activities of both P/CAF and p300/CBP. This inhibition is partially reversed by exogenously added P/CAF. These results imply that simultaneous recruitment of two distinct co-activators (p300/CBP and P/CAF) by Tax is essential for the assembly of a trans-activation competent, nucleoprotein complex.

The human T-cell lymphotropic virus, type-1 (HTLV-1),[1] is the etiological agent of adult T-cell leukemia/lymphoma and a neurodegenerative disorder, known as HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP 1–4). Viral infection leads to profoundly dysregulated cellular gene expression and concomitant T-cell proliferation. The uncontrolled proliferative effects, as well as neoplastic transformation by HTLV-1, are thought to be mediated by the viral trans-activator Tax (5, 6). Numerous cellular transcription factors, including NF-κB, SRF, AP-1, and CREB/ATF-1 family members, are aberrantly affected by Tax (7–22); effects on cell-cycle regulatory molecules and certain tumor suppressors have also been observed (23–29). HTLV-1 LTR trans-activation requires the assembly of the 40-kDa trans-activator Tax and CREB/ATF-1 transcription factors on three, 21-bp repeat enhancers located in the U3 region (22, 30–34). Tax is known to directly interact with the basic domain leucine zipper (bZip) of CREB/ATF-1, which binds the core cyclic AMP-responsive element (CRE) in each 21-bp repeat (7, 13, 21, 35). Recent data suggest that, upon binding to the basic domain of CREB bZip, Tax makes additional contacts with the G/C-rich sequences that flank the CRE; thus achieving the exquisite DNA sequence specificity of Tax-mediated LTR trans-activation (35–38).

Kwok *et al*. (39) have shown that the co-activator, CREB-binding protein (CBP), and its homologue, p300, directly bind to HTLV-1 Tax. We and others have confirmed and extended these results, and have demonstrated that amino acid residues 81–95 in Tax mediate its interaction with p300/CBP (40). Interestingly, the p300/CBP-binding region of Tax shares amino acid sequence similarity with residues comprising the kinase-inducible domain of CREB that undergoes Ca^{2+}-dependent Ser-133 phosphorylation in response to protein kinase A, MSK-1, or CAM-kinase IV activation (40–44). Mutation, or deletion, of these residues abolishes the ability of Tax to bind, or to form higher-order multiprotein complexes with p300/CBP *in vitro*, and impairs Tax-dependent trans-activation *in vivo* (40). We and others have also demonstrated that p300/CBP binding greatly stabilizes the activator-enhancer complex (39, 40, 45, 46). Interestingly, biochemical analyses of a well characterized Tax mutant, M47, indicate that it retains the ability to interact with CREB, 21-bp repeats, and p300/CBP, yet remains comparatively defective in trans-activation of the HTLV-1 LTR (40, 47, 48). These results suggest that recruitment of p300/CBP to the Tax/CREB/21-bp repeat complex is necessary, but not sufficient, for Tax-dependent trans-activation. Thus, interactions between Tax and additional cellular co-activators or general transcription factors are likely to be essential.

p300/CBP are general integrators of signal-dependent transcription; a diverse array of enhancer-binding factors utilize these co-activators for transcriptional activation in response to extracellular stimuli (49–53). In addition to their role in facilitating interactions between activators and components of the basal transcription machinery, p300/CBP have been shown to possess intrinsic histone acetyltransferase (HAT) activity (54).

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¶ To whom correspondence should be addressed. Tel.: 301-295-9624; Fax: 301-295-1545; E-mail: giam@bob.usuf2.usuhs.mil.

[1] The abbreviations used are: HTLV-1, human T-cell lymphotropic virus type-1; CRE, cyclic AMP-responsive element; ATF-1, activating transcription factor-1; CREB, CRE-binding protein; CBP, CREB-binding protein; P/CAF, p300/CBP-associated factor; HAT, histone acetyltransferase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis; LTR, long terminal repeat; bp, base pair(s); CAPS, 3-(cyclohexylamino)propanesulfonic acid.

Remarkably, another HAT, the p300/CBP-associated factor (P/CAF), has been shown to directly interact with p300/CBP to form a multi-HAT/activator-enhancer complex (55–57). The significance for the assembly of multiple-HATs on enhancer elements is a subject of intense investigation. Interestingly, the acetyltransferase activity of P/CAF is targeted toward histones H3 and H4 and appears to be redundant in light of similar activities of p300/CBP (57, 58). It has previously been suggested that this redundancy might reflect synergistic, or differential, HAT roles of these co-activators on certain promoters (57–61). Here, we provide evidence that recruitment of both p300/CBP and P/CAF by HTLV-1 Tax to the activator-21-bp repeat enhancer complex is essential for efficient LTR-dependent trans-activation. Moreover, we show that Tax-mediated LTR trans-activation is competitively inhibited by co-expressing the E1A 12S gene product, which inactivates both P/CAF and p300/CBP (57, 62–66). Tax-derived mutants, defective for direct interactions with either of these co-activators, *in vitro* and *in vivo*, are similarly defective in their abilities to activate transcription. These observations further imply that Tax might influence nuclear P/CAF-containing complexes; thereby potentially contributing to the pleiotropic dysregulated expression of numerous cellular genes during leukemogenesis.

EXPERIMENTAL PROCEDURES

Transfections and Reporter Gene Assays—HeLa cells (ATCC) were plated at 2×10^5 cells/60-mm dish and cultured for 24 h at 37 °C and 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin (Life Technologies, Inc.). These cells were washed twice with serum-free medium and transfected using liposome-mediated DNA transfer (LipofectAMINE reagent, Life Technologies, Inc.) as recommended by the manufacturer. Briefly, the cells were incubated for 4 h with the DNA/liposome mixtures; immediately following the incubation period, the transfection solution was removed, cells were washed with serum-free medium, and 2.5 ml of Dulbecco's modified Eagle's medium, supplemented with 20% fetal bovine serum and 100 μg/ml streptomycin sulfate, 100 units/ml penicillin, was added to each dish. The transfected cells were incubated for another 48 h and harvested for CAT-reporter assays by scraping, or extracted for immunoprecipitation. To control for variation in transfection efficiencies, cells were transfected in parallel with a CMV-lac Z expression vector and stained with 5-bromo-4-chloro-3-indoyl β-D-galactoside for β-galactosidase expression. CAT-reporter assays were carried out as previously reported (40). Results were quantified from triplicate experiments using an EAGLE-EYE II Video-imaging system (Stratagene, Inc.).

Co-immunoprecipitations and Western Blotting—Co-immunoprecipitations were carried out using extracts prepared from HeLa cells, transfected as described with a CMV-driven, FLAG epitope-tagged (f)P/CAF expression vector and either a CMV-driven wild-type or mutant, Tax expression plasmid. Transfected cells were harvested by scraping, washed three times with phosphate-buffered saline, and lysed in 500 μl of RIPA buffer (0.15 M NaCl, 50 mM Tris-Cl, pH 7.4, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% SDS), containing 50 ng/ml each of the protease inhibitors, pepstatin, leupeptin, chymostatin, bestatin, antipain-dihydrochloride (Roche Molecular Biochemicals), by repeated passage through a 27.5-gauge syringe. Cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4 °C. Two-hundred and fifty microliters from each sample was used for immunoprecipitation. Lysates were precleared by incubation with either 20 μl of a 50% slurry of Protein A- (Pierce, Inc.) or Protein G-agarose (Life Technologies, Inc.) and 5 μl of antiserum for 30 min at 4 °C, followed by brief centrifugation at 1200 rpm for 5 min. After preclearing, either 100 μl of an anti-Tax, monoclonal antibody (4C5) against a C-terminal epitope (located after residue 324) in Tax,[2] or 10 μl of anti-FLAG-M2, mouse monoclonal antibody (Eastman-Kodak Corp.), were added to each sample. The reactions were preincubated at 4 °C for 1 h; then, 100 μl of either protein A-agarose, or Protein G-agarose, were added to reactions containing anti-Tax or anti-FLAG-M2 antibodies, respectively, and further incubated overnight. On the following day, immune complexes were pelleted by centrifugation at 1200 rpm for 5 min at 4 °C, washed three times with 500 μl of RIPA buffer containing the above mentioned protease inhibitors, and resuspended in 30 μl of SDS-PAGE loading buffer. Samples were heated to 95 °C for 3 min, centrifuged, and 15 μl from each were loaded on a 12.5% SDS-polyacrylamide gel containing a 4% stacking gel. Electrophoresis was carried out at 150 V/150 mAmps using a 0.25 M Tris, 0.19 M glycine, 0.1% SDS running buffer; gels were transferred through a 10 mM CAPS, pH 11.0, 10% (v/v) methanol buffer to nitrocellulose membranes (Schleicher and Schuell, Inc.), and used for immunoblotting by standard protocols. For detection of (f)P/CAF expression, blots were incubated for 2 h with anti-FLAG-M2 antibody (diluted 1:1000), washed twice with BLOTTO buffer (50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.2% (v/v) Nonidet P-40, 0.02% (w/v) sodium azide, 5% nonfat dry milk), incubated for 1 h in an anti-mouse horseradish peroxidase-conjugated, secondary antibody (diluted 1:1000, Santa Cruz Biotechnology, Inc.), washed twice with BLOTTO, and once again with phosphate-buffered saline, and developed using a chemiluminescent substrate (SuperSignal, Pierce, Inc.). For detection of HTLV-1 Tax, or Tax-derived mutant proteins, the nitrocellulose membranes were probed with the anti-Tax monoclonal antibody (diluted 1:20) and immunoblotted as described. X-ray film was briefly exposed for autoradiography as previously mentioned. Alternatively, co-immunoprecipitations were performed using extracts prepared either from Jurkat and HTLV-1 transformed cell-lines (MT-4 and C8166), or JPX-9 cells, which express a metal-inducible version of wild-type Tax, induced for 12 h with 0, 1.5, 15, or 30 μM $CdCl_2$ in RPMI medium supplemented with 20% fetal bovine serum and 100 μg/ml streptomycin sulfate and 100 units/ml penicillin (Life Technologies, Inc.) in the presence of 10% CO_2. Extracts were prepared and treated as described previously, with the exception that immunoprecipitations were performed using 5 μl of a goat polyclonal, anti-P/CAF antibody (Santa Cruz Biotechnology, Inc.), and Tax was detected in P/CAF-containing complexes by Western blotting.

Plasmid Constructions and Protein Purification—The RcCMV plasmid, CMV-(f)P/CAF, CMV-E1A 12S, CMV-E1A 12SΔN, CMV-HTLV-1 Tax expression vectors, the CMV-driven expression vectors for the Tax-derived mutants, M47, K88A, and V89A, 218-CAT reporter plasmid, GST-HTLV-1 Tax, and GST-M47 vectors have all been previously described (22, 40, 47, 57, 67). pGEX-2T is from Amersham Pharmacia Biotech; and CMV-*lac Z* is from Life Technologies, Inc. GST-K88A and GST-V89A were constructed in GST-Tax by replacing the wild-type Tax coding sequence with those of the respective alanine substitution mutants (22, 40). GST fusion proteins were expressed and purified as prescribed by standard protocols. The purification of HTLV-1 Tax-His, or M47-His6, K88A-His6, and V89A-His6 have been previously described (22, 35, 40, 48, 67). Purification protocols for baculovirus-expressed p300 and FLAG epitope-tagged (f)P/CAF have likewise been reported elsewhere (54, 57).

Glutathione S-Transferase Binding Assays and in Vitro Complex Formation—To examine the possibility that HTLV-1 Tax and P/CAF might directly interact, 0.5–1 μg of purified GST or GST-Tax fusion protein (or an equivalent amount of each GST-Tax mutant protein) was pre-immobilized on 60 μl of a 50% slurry of washed glutathione-Sepharose 4B (Amersham Pharmacia Biotech) in 200 μl of 1 × binding buffer (25 mM Hepes, pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 10% (v/v) glycerol, 0.25 mM dithiothreitol) on ice for 2 h. The bound matrices were washed twice with 500 μl of 1 × binding buffer, pelleted by centrifugation at 1200 rpm for 5 min, and resuspended in 30 μl of 1 × binding buffer. Purified, FLAG epitope-tagged (f)PCAF (0.2–0.5 μg) was added to each binding reaction and samples were incubated for 30 min, with agitation, at room temperature. Following incubation, the bound matrices were washed three times with 500 μl of 1 × binding buffer and pelleted by centrifugation, resuspended in 30 μl of SDS-PAGE loading buffer, heated to 95 °C, and 15 μl from each reaction were analyzed by 12.5% SDS-PAGE and immunoblotting, as described.

To examine the recruitment of P/CAF and p300 by HTLV-1 Tax into a multiprotein complex bound to the 21-bp repeat DNA, we labeled 1 μg of the annealed oligonucleotides: 5′-GATCT**GGGCGTTGACGACAACCCCTC**ACCTCAAAAAACTTTC-3′ and 5′-TTTGAAAGTTTTTTGAGGT**GAGGGGTTGTCGTCAACGCCC**AGATC-3′ (HTLV-1 21-bp repeat sequence is shown in bold), with biotin-14-dATP (Life Technologies, Inc.) using 7.5 units of Klenow (New England Biolabs, Inc.) at 37 °C for 30 min. Labeled oligonucleotides were electrophoresed and purified from a 7.5% TBE (Tris borate/EDTA) acrylamide gel, eluted in 250 μl of deionized dH_2O, quantified, and 10 μl (20 ng) were used in each binding reaction. Sixty microliters of a 50% slurry of washed, streptavidin-agarose (Life Technologies, Inc.) were mixed with

[2] Y. Yao, Y-L. Kuo, Y. Tang, R. Harrod, C. Broder, W. Harrington, and C-Z. Giam, manuscript in preparation.

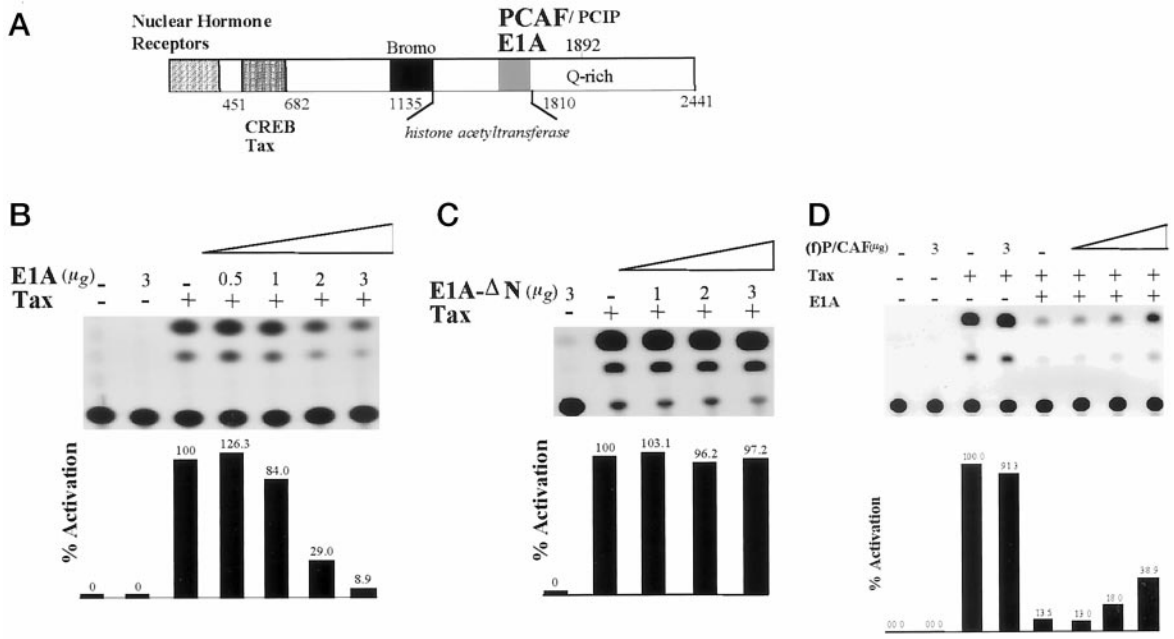

FIG. 1. **P/CAF and adenoviral E1A 12S share overlapping docking sites on the co-activator CBP.** *A*, diagram showing binding sites for E1A 12S and P/CAF (57, 59, 63, 65). *B*, Tax-dependent HTLV-1 LTR trans-activation is competitively inhibited by E1A 12S expression. HeLa cells were co-transfected with a 218-CAT reporter plasmid (1 μg), RcCMV empty vector, CMV-Tax (1.5 μg) and CMV-E1A 12S (0.5, 1, 2, and 3 μg) expression constructs; cells were harvested and chloramphenicol acetyltransferase activities were determined by radioisotopic assays. *C*, an E1A 12S mutant lacking the p300-interacting domain failed to compete against HTLV-1 LTR trans-activation. HeLa cells were transfected as described, with the exception that a mutant E1A 12S expression construct, CMV-E1A 12SΔN, was used (1, 2, and 3 μg). *D*, E1A 12S-competition of Tax-dependent LTR trans-activation is partially blocked by increasing intracellular P/CAF. HeLa cells were co-transfected with a 218-CAT reporter plasmid (1 μg), CMV-E1A 12S (3 μg), and CMV-Tax (1.5 μg) expression constructs. Increasing concentrations of a CMV-(f)P/CAF expression vector (0.5, 1.5, and 3 μg) were also included in certain reactions. Following 48 h incubation, cells were harvested, lysates prepared, protein concentrations quantified, and radioisotopic CAT assays were performed.

20 ng of biotinylated oligonucleotide in 200 μl of 1 × binding buffer (see above) and preincubated on ice for 2 h. The bound matrices were washed twice with 500 μl of 1 × binding buffer, pelleted by centrifugation, and resuspended in a total reaction volume of 50 μl (1 × binding buffer). One microgram of purified CREB bZip, 200 ng of HTLV-1 Tax-His6 (or Tax-derived mutant), 500 ng of baculovirus-expressed p300, and 500 ng of (f)P/CAF was added to each binding reaction and incubated at room temperature, with agitation for 30 min. Following incubation, the matrices were washed three times (5 min each) with 500 μl of 1 × binding buffer, pelleted, and resuspended in 40 μl of SDS-PAGE loading buffer. Ten microliters from each sample were heated at 95 °C for 3 min and loaded either on a 12.5% SDS-polyacrylamide gel, or a pre-cast 4–20% Tris glycine acrylamide gel (Novex, Inc.), for resolution of bound products. After electrophoresis, the gels were either transferred to nitrocellulose membranes for immunoblotting (Tax, P/CAF, p300) as described, or stained with Coomassie Blue reagent for direct visualization (CREB bZip). Purified p300 was detected using a monoclonal anti-p300 antibody (diluted 1:1000, Upstate Biotechnology, Inc.); P/CAF was detected using a goat anti-P/CAF polyclonal antibody (diluted 1:1000, Santa Cruz Biotechnology, Inc.), and an horseradish peroxidase-conjugated anti-goat secondary antibody (diluted 1:1000, Santa Cruz Biotechnology, Inc.).

RESULTS

E1A 12S Inhibition of Tax-dependent LTR Trans-activation—We have previously shown that the recruitment of p300/CBP alone by HTLV-1 Tax is necessary but not sufficient for optimal trans-activation. In this study, we examine the role of the co-activator/histone acetyltransferase, P/CAF, and its cooperation with p300/CBP in Tax-mediated trans-activation from the HTLV-1 LTR. It is well documented that P/CAF and the adenoviral E1A 12S protein share overlapping binding sites on the co-activator p300/CBP (Fig. 1*A*) (57, 59, 63, 65), and E1A 12S directly binds P/CAF and inhibits its transcription function (66). It is, therefore, possible that E1A 12S might negatively affect Tax-dependent trans-activation of a CAT reporter whose expression is driven by the two promoter-proximal 21-bp repeats of the HTLV-1 LTR. As shown in Fig. 1*B*, Tax-mediated trans-activation of a (×2) 21-bp repeat-CAT-reporter gene (218-CAT) was inhibited in the presence of increasing concentrations of a CMV-E1A 12S expression vector while the control RcCMV vector had no effect on Tax trans-activation. A vector that expressed a mutant form of E1A 12S, CMV-E1A 12SΔN, which lacks N-terminal amino acid residues necessary for its interaction with p300/CBP, did not significantly inhibit Tax-mediated trans-activation when transfected at levels comparable to that of the wild-type E1A 12S expression plasmid (Fig. 1*C*). These data are consistent with previous reports on E1A 12S inhibition of P/CAF-dependent transcription and further implicate P/CAF as an essential member of the functional activator-enhancer complex on the HTLV-1 21-bp repeats (57, 59, 63, 65, 66).

P/CAF Co-expression Partially Reverses E1A 12S Inhibitory Effects—To address whether E1A 12S inhibition of Tax-dependent LTR trans-activation occurs due to E1A 12S inactivation of a limiting pool of P/CAF, we attempted to reverse the E1A 12S inhibitory effect by exogenously increasing P/CAF expression. An inhibitory level of a CMV-E1A 12S expression vector was co-transfected with CMV-Tax and 218-CAT; a CMV-(f)P/CAF construct was included at progressively increasing concentrations in transfections. As shown in Fig. 1*D*, E1A 12S strongly inhibited Tax-mediated trans-activation from the 21-bp repeats, whereas, CMV-(f)P/CAF alone did not significantly affect basal, or Tax-dependent, reporter gene expression. Notably, E1A 12S inhibition was partially prevented by increasing the intracellular pool of (f)P/CAF. A complete restoration of Tax-dependent trans-activation, however, could not be achieved, even with relatively high CMV-(f)P/CAF concentrations. This is consistent with reports by others of a multi-level E1A 12S inhibitory effect on transcription (59, 63, 65, 66).

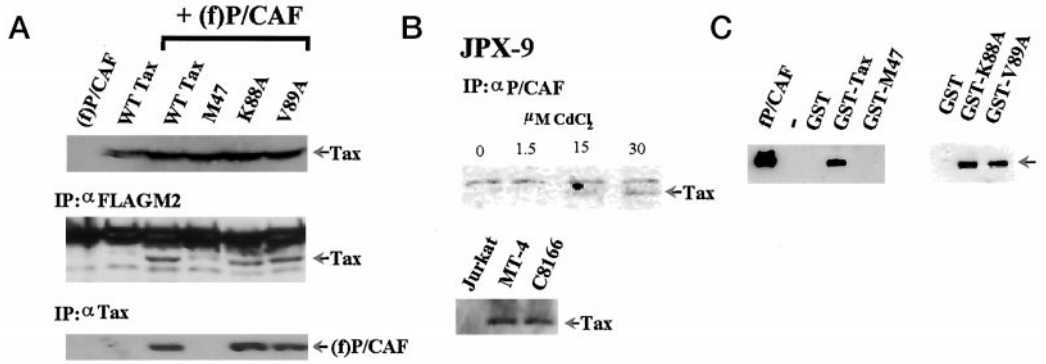

FIG. 2. **Wild-type Tax and mutants K88A and V89A interact with P/CAF in immunoprecipitation and GST pull-down experiments.** *A*, HeLa cells were co-transfected with CMV-(f)P/CAF (2 μg), and RcCMV empty vector, CMV-WT Tax, CMV-M47, CMV-K88A, or CMV-V89A (1.5 μg) as described. Cells were lysed and immunoprecipitation was performed as described under "Experimental Procedures." *B*, Tax is detectable in P/CAF immunocomplexes isolated from Tax-expressing lymphoid cells. Extracts were prepared from JPX-9 cells, treated with increasing concentrations of $CdCl_2$ to induce Tax expression, or from Jurkat and HTLV-1 transformed cell-lines (MT-4; C8166). Immunoprecipitations were performed as described under "Experimental Procedures." *C*, HTLV-1 Tax and p300/CBP-binding defective, Tax-derived mutants interact with (f)P/CAF in GST pull-down assays. Bound (f)P/CAF, following GST pull-down assays, was determined by immunoblotting using anti-FLAG-M2 antibody as described. One-third of the input (f)P/CAF is shown in the *far left lane*.

These data, collectively, are in agreement with the notions that P/CAF is a nuclear factor that is essential for HTLV-1 21-bp repeat trans-activation.

Analyses of Tax-P/CAF Complexes by Immunoprecipitation and GST Pull-down—In order to observe potential Tax-P/CAF interactions *in vivo*, HeLa cells were co-transfected either with a CMV wild-type, or mutant, Tax expression vector and CMV-(f)P/CAF, which expresses FLAG epitope-tagged P/CAF. Whole cell extracts were prepared as described and immunoprecipitation was performed using either an anti-FLAG-M2 antibody (Eastman-Kodak Corp.) or an anti-Tax monoclonal antibody. Immunoblotting results revealed that the wild-type Tax protein, as well as the CBP-binding defective mutants K88A and V89A, co-immunoprecipitated with (f)P/CAF using either the anti-FLAG-M2 antibody (Fig. 2*A, middle panel, lanes 3, 5*, and *6*) or anti-Tax antibody (Fig. 2*A, lower panel, lanes 3, 5*, and *6*). In contrast, the M47 mutant of Tax only weakly immunoprecipitated in complexes that contained (f)P/CAF (Fig. 2*A, top* and *middle panels, lane 4* of each). The minute but detectable amount of immunoprecipitated M47 most likely represents M47 indirectly complexed with (f)P/CAF through binding p300/CBP. Approximately equivalent amounts of wild-type, or mutant, Tax proteins and (f)P/CAF were expressed in transfected cells (Fig. 2*A, top panel*, and data not shown, respectively). To detect Tax-P/CAF interactions in Tax-expressing lymphoid-derived cells, JPX-9 cells were induced by treatment with increasing concentrations of $CdCl_2$ for 12 h; extracts were prepared and immunoprecipitation was performed as described (68). Upon induction, Tax was detectable in P/CAF-containing immune complexes in a dose-dependent manner (Fig. 2*B, top panel*). In addition, Tax could also be immunoprecipitated with P/CAF in extracts prepared from HTLV-1-transformed cells (Fig. 2*B, bottom panel*). The presence of approximately equivalent amounts of P/CAF in these immunoprecipitates was verified by Western blotting (data not shown). These results suggest that HTLV-1 Tax most likely complexes directly with P/CAF.

In an effort to biochemically analyze Tax-P/CAF interactions *in vitro*, GST pull-down assays were performed using purified (f)P/CAF and immobilized GST wild-type, or mutant, Tax proteins. As shown in Fig. 2*C*, GST-Tax, GST-K88A, and GST-V89A all displayed efficient binding to (f)P/CAF. The GST-M47 protein, however, did not show a significant interaction with (f)P/CAF; neither did the matrix alone or GST control. Input levels of purified GST or GST-Tax fusion proteins were comparable as determined by SDS-PAGE and Coomassie staining. Again, in this assay, the M47 mutant of Tax is defective in its ability to interact with P/CAF *in vitro*; reflective of data obtained from immunoprecipitations and transfection experiments as described earlier. Of note, P/CAF co-expression could not significantly restore the trans-activation defect observed for M47 *in vivo* (data not shown).

Assembly of a Multi-HAT/Activator-Enhancer Complex—In order to study the recruitment of P/CAF, as well as p300/CBP, by HTLV-1 Tax on the 21-bp repeat*, in vitro*, we formed multiprotein complexes in the presence of purified CREB bZip on immobilized, biotin-labeled 21-bp repeat oligonucleotides. As shown in Fig. 3, approximately equivalent amounts of CREB bZip and biotinylated-DNA were eluted from each reaction. Likewise, similar amounts of HTLV-1 Tax, or Tax-derived mutants M47, K88A, and V89A, were observed in complete binding reactions (*lanes 3–6*). No significant complex formation was observed in the negative controls, either in the absence of CREB bZip (*lane 1*) or Tax (*lane 2*). Consistent with previously published data, only wild-type Tax and M47 significantly interacted with purified p300 in multiprotein complexes on the 21-bp repeat DNA (*lanes 3* and *4*). Interestingly, P/CAF binding was observed only in complete binding reactions that contained wild-type Tax, K88A, or V89A (*lanes 3, 5*, and *6*). These results are in agreement with those obtained from co-immunoprecipitation and GST pull-down experiments, and further confirm the hypothesis that HTLV-1 Tax interacts directly with both P/CAF and p300/CBP in a multi-coactivator/activator-enhancer complex. Of significance, the M47 mutant (*lane 4*) did not exhibit higher order complex formation with P/CAF, even though its recruitment of p300/CBP appeared not to be impaired; thus, the p300-P/CAF interaction appeared weak under our assay conditions, requiring Tax to further stabilize multiple protein contacts.

V89A and M47 Exhibit Trans-complementation Properties—The importance of both p300/CBP and P/CAF interactions in Tax-mediated trans-activation is further suggested by the trans-complementation effect of the V89A mutant, which is defective in p300/CBP binding, on the trans-activation activity of M47, a mutant shown here to be defective in P/CAF binding. As shown in Fig. 4, V89A and M47 mutants each exhibited residual trans-activation activities. Co-expression of V89A and M47 partially restored the trans-activation activity of each mutant in a dose-dependent manner, possibly through the formation of a partially active M47/V89A heterodimer, as suggested by the molar ratios for optimal trans-activation. Furthermore, their combined effect appeared to be synergistic.

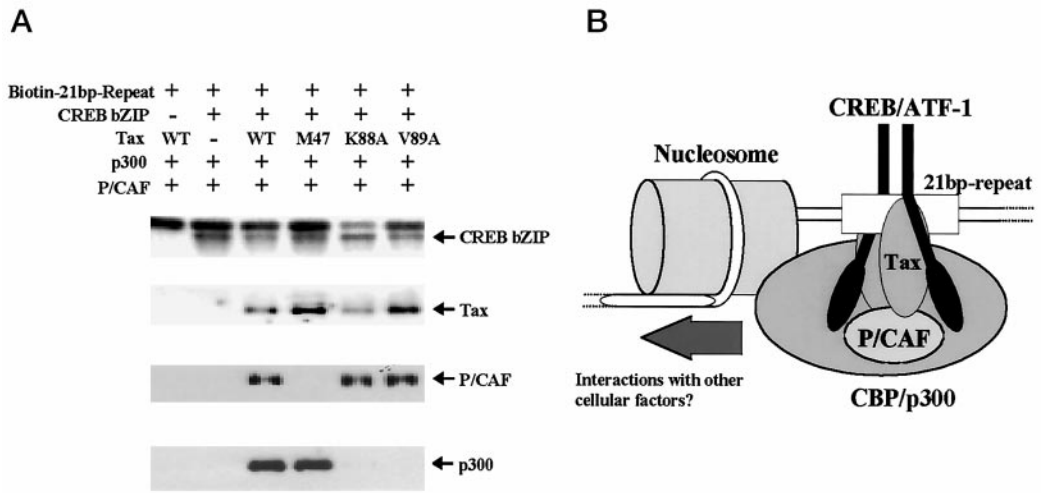

FIG. 3. **Assembly of a multi-HAT/activator-enhancer complex.** *A*, CREB bZip, Tax, or Tax-derived mutants, p300, and P/CAF proteins were incubated with a biotinylated 21-bp repeat DNA probe. Streptavidin-agarose affinity matrices were used to isolate bound components. After washing, bound factors were resolved by SDS-PAGE and visualized either by Coomassie staining or immunoblotting as described. *B*, model of interactions between Tax and p300-P/CAF on an HTLV-1 21-bp repeat element.

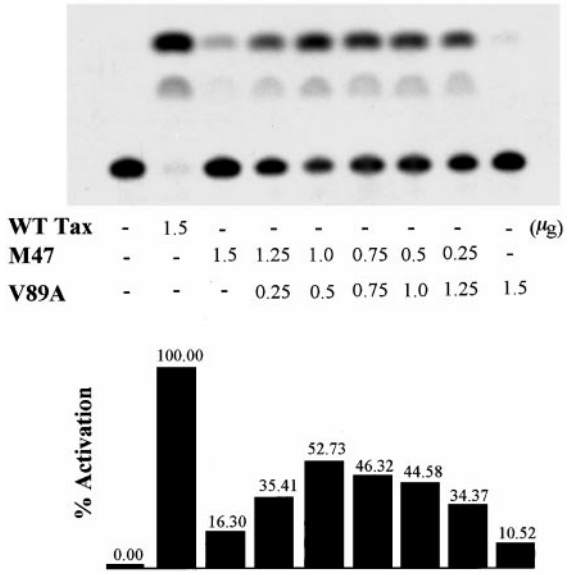

FIG. 4. **The V89A and M47 Tax mutants exhibit synergistic trans-complementation.** HeLa cells were co-transfected with a 218-CAT reporter plasmid (1 μg), CMV-Tax (1.5 μg), CMV-M47 (1.5, 1.25, 1.0, 0.75, 0.5, or 0.25 μg), and/or CMV-V89A expression constructs (1.5, 1.25, 1.0, 0.75, 0.5, or 0.25 μg). Cells were cultured, lysates were prepared, and CAT assays were performed and quantified as described.

These data are suggestive that a tripartite interaction between Tax, p300/CBP, and P/CAF, together with CREB dimer bound to the 21-bp repeat element, might be required in order for Tax-mediated LTR trans-activation to occur (Fig. 3*B*).

DISCUSSION

With this study, we have demonstrated that both P/CAF and p300/CBP directly interact with HTLV-1 Tax. Immune complexes containing P/CAF and Tax were readily detectable in extracts prepared either from induced JPX-9 cells or HTLV-1 transformed cell lines. Recruitment of both co-activators is required for Tax-mediated trans-activation from the HTLV-1 enhancer. P/CAF is a co-activator/HAT that has been shown to recognize a domain in p300/CBP that also interacts with the adenoviral E1A 12S protein (57, 59, 63, 65). Indeed, E1A 12S has been reported to competitively inhibit the function of activators, such as Stat-1a, by displacing P/CAF from p300/CBP (59, 60). Recently, others have also demonstrated that the E1A 12S protein can directly inhibit P/CAF-dependent activation (66). Consistent with the function of P/CAF as a key co-activator/HAT in specific enhancer complexes that contain p300/CBP, Tax-mediated LTR trans-activation was competitively inhibited by co-expression of E1A 12S. This inhibition was, at least partially, prevented by increasing the intracellular concentration of P/CAF. That a complete reversal of E1A 12S inhibition was not achieved is consistent with a multilevel inhibitory effect of P/CAF function by E1A 12S as proposed by others (59, 63, 66).

Although p300/CBP have been shown to directly bind P/CAF (57, 59), in our *in vitro* assays, the presence of Tax is critical for the recruitment of both p300/CBP and P/CAF, respectively. The M47 mutant, which efficiently interacts with p300/CBP, did not show a detectable interaction with P/CAF. The Tax mutants K88A and V89A, which were previously characterized as being defective for p300/CBP binding (40), were able to interact with P/CAF but unable to bind p300 in the biotin-21-bp repeat pull-down experiments. Thus, the p300/CBP-P/CAF interaction might be relatively weak under the conditions of our assay, and requires Tax to further stabilize protein contacts. The implications from these results may extend to promoter elements of other genes where multiple activator/co-activator interactions are needed to drive transcriptional activation. As both classes of Tax mutants, M47 and K88A/V89A, defective for P/CAF and p300/CBP binding, respectively, are impaired, but not completely abrogated, in their abilities to activate LTR-dependent transcription (40, 47), these data support the notion that recruitment of both types of co-activators to the HTLV-1 21-bp repeat by Tax is necessary for optimal trans-activation. Consistent with this hypothesis, P/CAF co-immunoprecipitated with wild-type Tax, as well as with p300/CBP-binding defective Tax mutants; likewise similar binding was observed in GST pull-down experiments *in vitro*. Conversely, M47, which interacts with p300/CBP with an affinity comparable to that observed for the wild-type Tax protein (40), neither exhibited significant interaction with P/CAF in immunoprecipitations, nor in GST pull-down experiments.

p300/CBP and P/CAF have previously been shown to possess histone and protein acetyltransferase activities (54, 57, 58). In addition, these proteins are known to interact with an increasing array of activators and general transcription factors (49–51). It is possible that the major function of p300/CBP and

P/CAF, upon their recruitment by Tax to the viral enhancer, is to modify histones to release the HTLV-1 promoter from the suppressive effects of chromatin architecture. Therefore, the interaction of HTLV-1 Tax with two distinct co-activator molecules might reflect a requirement for dual-HATs to remodel chromatin at the 21-bp repeat element via multistep, and possibly multisite, nucleosomal acetylation. Alternatively, P/CAF and p300/CBP may act at separate stages of transcription. Indeed, p300 is known to directly bind RNA polymerase II, whereas, P/CAF has been shown to interact only with the hyper-phosphorylated, elongation-competent form of the polymerase (69). It remains to be determined at precisely which stage, initiation or elongation, the trans-activation defects for M47 and K88A/V89A reside. Our present study suggests that interactions between Tax and other components of the cellular co-activator network, or basal transcription machinery, are likely to be necessary for efficient Tax-mediated LTR trans-activation.

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